Recommended Supplies for Microarray Labeling and Hybridization (*March 2005*)		
SUPPLIES	**SUPPLIER**	**Catalog No.**
SuperScript™ Indirect cDNA Labeling Kit	Invitrogen	L1014-02
Dyes: Cy3 monofunctional reactive dye Cy5 monofunctional reactive dye	 Pharmacia Pharmacia	 PA23001 PA25001
Ribonuclease H (RNase H)	Invitrogen	18021-071
Formamide, molecular biology grade, (deionized if available)	Any reputable chemical manufacturer	We recommend buying small aliquots, ≤ 100ml bottles
Coverslips for 48-pin print: Lifterslips™ (25 X 60 mm) or mSeries™ (25 X 60 mm)	 Erie Scientific Erie Scientific	 25X60I-2-4789 25X60I-M-5439
Staining Dish/rack (10 slide)	Fisher	08-812
Slide Box (100 slide)	Thomas Scientific	6708-G28
Slide Box (25 slide)	Thomas Scientific	6708-G08
Hybridization chambers: Dual Hyb Chamber Single Hyb Chamber Single Hyb Chamber Deeper hyb chamber to accommodate thicker mSeries™ cover slip: Single Hyb Chamber	 Genomic Solutions Telechem Int., Inc. Corning Telechem Int., Inc.	 JHYB200004 AHC 2551 AHCXD
Hyb Oven	Fisher Scientific	13-247-10
Forceps	Fisher Scientific	10-295
Mini-Elute PCR Purification Kit	Qiagen	28004
High Quality Pre-filtered BSA	Invitrogen	15561-020
Centrifuge with microplate carrier assembly		

Target Preparation/Hybridization Using Total RNA

I. cDNA Generation:

Prepare separate cDNA labeling reaction for each fluorescent dye you wish to use.
A "master mix" (step 4) can be made, and the reaction increased up to 5X if needed.

1. For each dye dilute total RNA to between 5.0 – 20.0 µg in 16.0µl of DEPC water (0.3- 1.25 µg/µl).
2. Add 2.0µl of 2.5µg/µl anchored oligo $d(T)_{20}$ primer.
3. Incubate at 70°C for 5 minutes. Cool on ice for at least 1 min.
4. Combine the following components for each sample in a sterile, RNase/Dnase-free microcentrifuge tube:
    a. 6.0µl of 5X First-Strand buffer
    b. 1.5µl of 0.1 M DTT
    c. 1.5µl of 10mM dNTP mix
    d. 1.0µl of RNaseOUT™ (40 U/µl)
5. Add the mixture to the annealed primer and RNA.
6. Add 2 µl of 400 U/µl SuperScript™ III RT and incubate at 48°C for 2hrs . (Final volume is 30µl)
7. Incubate at 70°C for 5 minutes to stop reaction.
8. Cool down by spinning in a microcentrifuge at maximum speed for 1 minute.
9. Add 2µl of 2 U/µl RNase H and incubate at 37°C for 20 min.
10. Add 0.5 µl of 0.5M, pH 8.0 EDTA, mix well and proceed with purification.

II. cDNA purification: (QIAGEN MINElute purification kit)

1. Add 200 µl of Binding buffer ***PB*** to each RT reaction and mix well.
    Note: recommended maximum is 2 RT reactions per column.
2. Apply each reaction to separate spin columns. Incubate for 1 minute.
3. Spin for 1 min at full speed.
4. Discard flow-through.
5. Add 500µl of Wash buffer ***PE*** per reaction (Be sure that ethanol was added to ***PE*** buffer).
6. Spin for 1 min at full speed.
7. Discard flow-through.
8. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
9. Place columns in a fresh 1.5ml microcentrifuge tubes.
10. Add 10µl of 1:10 Elution buffer ***EB*** directly to the membrane. (dilute elution buffer 1:10 with Molecular Biology Grade water).
11. Incubate for 1 min. at room temperature.
12. Spin for 1 min at full speed.
13. Add another 10µl of diluted ***EB*** buffer to the membrane.
14. Incubate for 1 min. at room temperature.
15. Spin for 1 min at full speed.
16. Dry down in SpeedVac for ~15 min at medium temp. DO NOT OVERDRY!

III. NHS-ester containing dyes coupling reaction:

1. Resuspend cDNA pellet in 5µl of 2x coupling buffer. (If pellet was over dried gently heat at 37° C for 15 minutes to aid in the resuspension process.)
2. The first time a tube of dye is used, resuspend in 45µl DMSO. Use DMSO provided with the kit.
3. Add 5µl of the resuspended monofunctional reactive dye to cDNA.
4. Mix thoroughly by gently pipetting up and down.
5. Incubate minimum for 30 minutes at room temp in the dark, flicking the tubes occasionally.

*IV. **Dye-Coupled cDNA Purification:** (using QIAGEN MINElute purification kit)*

1. Add 10µl of 3M Sodium Acetate, pH 5.2 to each labled cDNA reaction, mix well.
2. Add 200µl of Binding buffer ***PB*** to each reaction and mix well.
3. Apply each reaction to a separate spin column.
4. Incubate for 1 min. at room temperature.
5. Spin for 1 min at full speed.
6. Discard flow-through.
7. Add 500µl of Wash buffer ***PE*** per reaction (Be sure that ethanol was added to ***PE*** buffer).
8. Spin for 1 min at full speed.
9. Discard flow-through.
10. Repeat wash step.
11. Discard flow-through.
12. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
13. Place columns in a fresh 1.5ml microcentrifuge tubes.
14. Add 10µl of 1:10 diluted Elution buffer ***EB*** directly to the membrane
15. Incubate for 1 min. at room temperature.
16. Spin for 1 min at full speed.
17. Add another 10µl of 1:10 diluted Elution buffer ***EB*** directly to the membrane.
18. Incubate for 1 min. at room temperature.
19. Spin for 1 min at full speed.
20. Can read OD with the Nanodrop to determine labeling efficiency and cDNA concentrations.

*V. **Pre-hybridization:** (should start approximately 2 hours before setting up hybridization)*

Pre-hybridization buffer = 5X SSC, 0.1% SDS and 1% BSA. (Can make 10% BSA stock and filter before use or purchase pre-filtered BSA; store pre-hyb buffer at -20° C and thaw only once, warm to 42° C prior to use.)

1. Apply 80 µl of pre-hybridization buffer under a Lifterslip to the array and incubate for 42° C for at least 30 mins and up to 1 hour.
2. Wash off the pre-hybridization solution by rapidly plunging the slide in distilled water for 2 mins, then transfer slide to 100% isopropanol for 2 mins.
3. Allow slide to air dry completely prior to use or spin dry.

*VI. **Setting up hybridization:***

1. Combine Cy3 and Cy5 labeled targets together (~20µl recovered for each).
2. Denature target at 100^{o}C for 1 minute, then snap cool on ice. (Final volume should be about 40µl)
3. Make fresh 2X Formamide hybridization buffer (50% formamide, 10x SSC, 0.2% SDS) and warm to 42^{o}C just before adding to samples.
4. Add 20µl of water to wells in hybridization chamber to maintain humidity.
5. Add 40µl of 2X F-hyb buffer to samples
6. Load 80µl sample onto microarray under a Lifterslip.
7. Incubate overnight (12-16 hours) at 42° C in water bath or hybridization oven.

Wash:

- 2x SSC, 0.1% SDS for 2 minutes, with occasional plunging
- 1x SSC, for 2 minutes, occasional plunging
- 0.2x SSC, for 2 minutes, occasional plunging
- Spin 3 minutes / 650 rpm to dry

WASHES:	2XSSC+0.1%SDS	1XSSC	0.2XSSC
dH_20:	179 ml	190 ml	198 ml
20XSSC:	20 ml	10 ml	2 ml
20%SDS:	1 ml		